

A look into the cockpit of the developing locust: looming detectors and predator avoidance.

Abbreviated title: Development of LGMDs and hiding behavior

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ABSTRACT

For many animals, the visual detection of looming stimuli is crucial at any stage of their lives. For example, human babies of only 6 days old display evasive responses to looming stimuli (Bower et al., 1971). This means the neuronal pathways involved in looming detection should mature early in life. Locusts have been used extensively to examine the neural circuits and mechanisms involved in sensing looming stimuli and triggering visually-evoked evasive actions, making them ideal subjects in which to investigate the development of looming sensitivity. Two giant motion detecting (LGMD) neurons have been identified in the lobula region of the locust visual system: the LGMD1 neuron responds selectively to looming stimuli and provides information that contributes to evasive responses such as jumping and emergency glides. The LGMD2, responds to looming stimuli and shares many response properties with the LGMD1. Both neurons have only been described in the adult. In this study, we describe a practical method combining classical staining techniques and 3D neuronal reconstructions that can be used, even in small insects, to reveal detailed anatomy of individual neurons. We have used it to analyze the anatomy of the fan-shaped dendritic tree of the LGMD1 and the LGMD2 neurons in all stages of the post-embryonic development of *Locusta migratoria*. We also analyze changes seen during the ontogeny of escape behaviors triggered by looming stimuli, specially the hiding response.

Keywords: looming detection, LGMD, development, hiding, neuronal reconstruction

INTRODUCTION

The image of an approaching stimulus often signifies danger to an animal, and the ability to detect and respond appropriately to it can be crucial for survival. Looming-sensitive visual neurons are known in several animals (pigeon: Sun and Frost, 1998; fish: Preuss et al., 2006; fruit fly: Fotowat et al., 2009; frog: Nakagawa and Hongjian, 2010), and have been particularly well studied in locusts where they were first described (Schlotterer, 1977; Rind and Simmons, 1992). Locusts possess a pair of large and uniquely identified visual neurons, the lobula giant movement detectors, LGMD1 (known mostly as LGMD) and LGMD2, which respond selectively to the images of an object looming towards the eye (Rind, 1987; Simmons and Rind, 1997). Extensive research has been done on the circuitry involved in looming detection and evasive behaviors in adult locusts (Rind and Simmons, 1992; Simmons and Rind, 1992; Hatsopoulos et al., 1995; Gabbiani et al., 2002; Santer et al., 2005). The majority of this work has focused on the LGMD1 and on its postsynaptic neuron, the descending contralateral movement detector (DCMD; Rowell, 1971; Rind, 1984). However, additional neurons in the locust visual system also respond to looming stimuli, and have been implicated in triggering evasive responses (Gray et al., 2010; Fotowat et al., 2011). The LGMD2 shares many key features with the LGMD1, including selective responses to looming stimuli, but it has been less often studied (Rind, 1987, 1996; Simmons and Rind, 1997) and neither its role in behavior nor its postsynaptic target neurons have been explored. Almost all research on looming sensitive neurons has been done on adult locusts. Yet, in nature, locusts avoid predation throughout their lives. One of the earliest escape strategies locusts use, when sitting on a plant stem is hiding, by moving around the stem away from a looming stimulus (Hassenstein and Hustert, 1999). All juveniles as well as adults could perform this behavior although it has not been tested before. Because hiding is triggered by

looming stimuli, it could potentially involve the LGMD-DCMD system. The DCMD is already known to be involved in evasive behaviors: in controlling emergency glides and in different phases of the jump (Rind and Santer, 2004; Santer et al., 2005, 2006, 2008; Fotowat and Gabbiani, 2007).

Locusts follow a hemimetabolous pattern of development and grow gradually through post-embryonic development, so that, apart from the absence of functional wings, the juveniles share most of the physiological and morphological characteristics of the adults (Sehnal, 1985). The segmentally repeated pattern in the nervous system in hemimetabolous insects arises during early embryogenesis, as a stereotyped axonal scaffold upon which growth cones then fasciculate (Raper et al., 1983a, b). Synapses form, late in embryonic development (75%, Leitch et al., 1995) and within a localized region of neuropil (Bastiani et al., 1994). The final structure of each neuron is not completely specified in the embryo: in locusts, mechanosensory pathways established in the embryo can subsequently be modified during post-embryonic development, either by removal of the original target or by blocking activity (Pflüger et al., 1994). In the eye and optic lobe, where a continuous retinotopic map is established by the sequential ingrowth of receptor axons, precise connections are established gradually (Meinertzhagen, 1976). This gradual process extends throughout larval development in hemimetabolous insects and occurs rapidly during pupation which lasts 4 days in holometabolous ones like *Drosophila* (Heisinger et al., 2006). In the locust new ommatidia are added at the anterior margin of the eye at each moult and receptors grow in to the lamina (Anderson, 1978). New connections with the LGMD could either be formed between incoming neurons and newly extended fine distal LGMD dendrites, or by a competition for exiting space over an enlarged and fully branched neuron. In the last case, the main changes occurring during development would be an increase in the density of synapses

without an obvious change in the dendritic organization of the LGMD. In adults, most synapses are located on the fine processes of the LGMD1 and 2 (Rind and Simmons, 1998).

The stereotyped anatomy of the insect optic lobe, which has a high degree of invariance in identified neurons between different individuals, suggests that visual experience does not play a major role in shaping the circuits in the visual system. However, early visual experience has an effect on the size of the optic lobes in *Drosophila* (Barth et al., 1997) and synapse numbers in other flies (Rybak and Meinertzhagen, 1997). This effect is mainly at the level of the receptor cells, and in the lobula region of the fly optic lobe visual experience was found to have no influence on the anatomy of the directionally selective tangential cells (Karameier et al., 2001).

Studying the development of the DCMD neuron Bentley and Toroian-Raymond (1981) found that by 80% of embryonic development the adult arborization pattern of the DCMD was already established. Although the post-embryonic development of the LGMD1 neuron has not been studied directly, we have recently shown that the 1st instar DCMD neuron, postsynaptic to the LGMD1 is already able to respond to approaching objects although its selectivity and intensity of response both increase during postembryonic development (Simmons et al., 2013). Which changes in the LGMD1 dendritic tree structure accompany this improvement is not known.

Here, we decided to study in detail the changes undergone during post-embryonic development by two neurons with established relevance in looming detection. In adults, intracellular staining is normally used for morphological characterization of the LGMD neurons (O'Shea and Williams, 1974; Rind, 1987; Simmons and Rind, 1997; Rind and Simmons, 1998; Peron et al., 2007). Using the same methods in young instars would be difficult especially considering newly hatched locusts have heads about 1 mm long. Instead, we used a technical approach combining classical staining techniques and 3D neuronal

reconstructions that allowed the characterization of the anatomy of the LGMD1 and 2 neurons in the lobula throughout post-embryonic development, from the newly hatched 1st instar to the last larval instar, the 5th. We performed a morphometric analysis to evaluate the changes produced in the two neurons during post-embryonic development. We also describe the accompanying modifications, occurring through ontogeny, in the performance of escape responses shown by unrestrained locusts positioned on a rod, to either real, or computer generated, looming stimuli.

MATERIALS AND METHODS

We used male and female 1st to 5th larval instars and adults of *Locusta migratoria*, taken from a gregarious laboratory culture kept at 30°C, with a 12:12 hours light-dark cycle. Eggs were laid in sand-filled pots. Instar was determined by measuring the size of the body and wing buds and by comparing the shape of the pronotum with reference measurements we made in carefully staged locusts. First instar hoppers were used between 1 and 12 hours of hatching from the egg.

Neuronal reconstruction:

Intracellular staining in adult locusts has shown previously that both LGMD neurons are large, relatively invariant in shape between individuals and occupy a relative consistent position in the lobula (Rind and Simmons, 1998; Rind and Leitinger, 2000; Peron et al., 2007). In toluidine blue stained sections through the brain, LGMD1 and LGMD2 are readily recognizable in the distal lobula since their processes are considerably wider in diameter than those belonging to other neurons. They are arranged in two crescents, the processes of the LGMD2 located nearer to the posterior brain surface than those of the LGMD1 (Rind and

Leitinger, 2000). Accordingly, the lobula arborizations of the two neurons were easily recognized and distinguished in reduced silver preparations in all instars as well as in adults (Fig 1A). We used Bodian's reduced-silver method (following the protocol described in Sztarker et al., 2005). Briefly, after cold anesthesia, locust's heads were cut off, mandibles removed and a window opened in the cuticle in the back of the head to allow rapid fixation. Brains were fixed for 6 hours in AAF (5% glacial acetic acid, 85% ethanol, 10% formalin). After washing, brains were dissected out of the head, dehydrated and mounted in Paraplast plus (TAAB laboratories Equipment, Berkshire). Blocks were serially sectioned at 12 μ m. Dewaxed sections were incubated overnight at 45°C in 250 ml distilled water containing 1% silver proteinate (Johnson Matthey SA, London) and 1-2 g clean copper turnings (Fisher Scientific UK, Leicestershire) per 100 ml solution. After incubation, tissue was placed in developer, gold toned, reduced, and then fixed. Following dehydration, sections were mounted in Histomount (Fisher Scientific UK, Leicestershire) under coverslips. LGMD profiles were identified using an Olympus BH-2 microscope and then were traced in several successive 12 μ m sections (4 to 12 sections depending on animal size) using Neurolucida software (MBF bioscience, Williston, VT) as detailed in the results section to generate three-dimensional reconstructions of the neurons.

We used NeuroExplorer (MBF bioscience, Williston, VT) to analyze structural changes in the LGMD1 and LGMD2 neurons during post-embryonic development. We quantified the length and the number of segments constituting each dendritic tree. Then, by analyzing the branch order of each segment we obtained the maximum branch order and plotted branching pattern histograms for the frequency of each order of branch. We additionally analyzed the data derived from the segment analysis of the dendritic tree structures of LGMD1 and LGMD2 neurons using a Canonical Discriminant Analysis (CDA) in four of the five instars using SPSS v19 (IBM Statistics, Armonk, NY). We used the anatomical details of 15 LGMD

neurons in total, 8 LGMD1 and 7 LGMD2: 2 LGMD1 and 2 LGMD2 from each of the 1st and 2nd instars; 3 LGMD1 and 2 LGMD2 from the 4th instars and 1 LGMD1 and 1 LGMD2 from 5th instar locusts.

In some subjects data was from the same individual, in others, as in the 1st instar, LGMD1 and 2 were from separate individuals but were closely matched in size as these were 1 day old (3rd instars were not included since there were size differences between the individuals from which LGMD1 and LGMD2 were reconstructed). We used this analysis to quantify the changes produced in each of these neurons during development and to identify which structural parameters could be used to categorize them. It also provided a distance measure to have a quantitative index of similarity. We tested the following metrics, defined as in figure 1: x, y and z co-ordinates of each branch point, the order of each branch in the dendritic tree, the change in xy and z angle of each branch compared with the lower order branch, from which it arises (the last two represent the spherical coordinate angles for the end of a segment relative to the start of the next segment). Coordinates reflect the shape of each neuron and relate to natural head and body axes: z corresponds to the anterior-posterior axis; x to the dorso-ventral and y to the proximal-distal. Because the LGMD2 is located posteriorly to the LGMD1, nearer to the hind surface of the optic lobe, when both neurons were reconstructed from the same optic lobe the z values of the LGMD 2 are more positive than those for the LGMD1.

Hiding behavior:

For our initial series of behavioral experiments we used a real object looming towards the animal to elicit escape responses. The looming stimulus consisted of a 10 cm diameter black Styrofoam ball approaching along a 1 meter rail (inclination 2.2°). The approach took 2.3 sec, with an initial object subtense on the eye of 5.4°, a final subtense of 79.6° giving an overall 1/v value of 115 msec, assuming a constant velocity of 0.44m/s. In fact the ball rolling down

an incline will be subject to acceleration due to gravity (0.38 m/s^2), calculated using uniform accelerated rectilinear motion formulas, giving a final velocity of 0.874 m/s . We selected a slow approach to bias responses towards hiding. Faster approaching stimuli (steeper inclination) produced a higher number of jumping responses.

Two metal rings with a motion detector attached documented the passing of the ball just after the release and near the end of the trajectory (1 meter apart, white arrowheads in Fig 5A). When the ball passed each detector an LED was illuminated. The two LEDs were filmed along with the locusts to synchronize the response with the passage of the stimulus. The ball was held in place and then released by a long stick that was moved gently (Fig 5B), with a movement that did not trigger any response from the locust in the absence of a ball. The locust sat on a vertical wooden rod in a head-up posture. We used a different rod size for each instar, maintaining a similar proportion between the size of animal and rod (diameter: 2.5 mm for 1st and 2nd instars, 4 mm for 3rd, 6 mm for 4th, 7 mm for 5th and 9 mm for adults). Animals were unrestrained. They were placed perpendicular to the trajectory of the ball and viewed the ball only with the right eye. Locusts were first left undisturbed for 5 minutes and then three trials, separated by 5 minutes intervals, were conducted with each animal. Responses were filmed from above with a JVC GR-D290EK digital video camera at a resolution of 30 frames/sec and analyzed off-line.

After classifying the response in a video recording (see results), videos were analyzed frame by frame using the program 'Imaging MotionScope' (Redlake, San Diego, CA). We determined: a) the frame before the one in which movement of the locust was first detected (initial position, IP); b) the frame in which the furthest rotational position relative to the original position was reached (FP, final position); c) the latency of response, calculated as the time between the ball passed through the first metal ring (the first LED turned on) and the IP; d) the angle through which the locust had rotated, calculated using the program ImageTool

(UTHSCSA, San Antonio, TX) to measure the angle formed by the positions of the midline of the locust on the frames IP and FP relative to the centre of the rod; and e) the velocity, calculated as the angle rotated during the first 66 to 200 ms of the response.

Locusts that repeatedly jumped as a response were discarded (less than 5% of the animals). This stimulus was very effective in generating behavioral responses in locusts (we recorded active responses in 99.5% of the trials).

For our second series of behavioral experiments, we used computer-generated stimuli to trigger hiding in locust. We used different combinations of velocities (0.25; 0.5; 1; 1.5 ms⁻¹) and sizes (8.5; 15 cm diameter) to stimulate 5th instar locusts and selected a 15 cm diameter dark disk that loomed at 0.25 ms⁻¹, starting 1.55m from the locust as the more effective for triggering hiding responses. This gave an approach with an $l/|v|$ ratio of 300 msec, where l is object radius and $|v|$ is approach velocity (Gabbiani et al., 2002). We used dark and light stimuli. The contrast of the looming stimulus against the background, defined as (object intensity-background intensity)/background intensity, was 0.8 (background illuminance, measured at the screen with a 'Tecpel 530' light meter was 265 lux; the dark disk had an illuminance of 74 lux and the light disk of 456 lux). A locust sat vertically on a rod 6 cm from the screen and viewed the image expansion with the right eye. Stimuli were displayed on a 20 inch, Clinton Monoray monitor from Cambridge Research Systems, with a fast greenish-yellow phosphor (DP104) and a refresh rate of 150 Hz. Looming stimuli were generated using a Cambridge Research Systems VSG2/5B graphics card in a Dell Optiplex GX260 computer. We used 1st, 3rd and 5th instars (10 locusts in each group). Each animal was left undisturbed for 5 minutes and then was subject to six trials separated by 3 minutes, three with a dark and three with a light disk, presented in a random order. Responses were filmed from above and analyzed off-line as before. For timing the responses, the end of the stimulus

approach could be seen in the videos. Some additional trials (not used for analyzing locust responses) were recorded in which a mirror enabled us to capture the complete stimulus dynamic into the recorded frame.

We performed a control experiment with 9 additional 3rd instars to verify that the locusts were responding to the actual loom and not to the accompanying luminance change. We stimulated each locusts with a set of three or more trials with the dark looming stimulus, then a set of three trials with static equivalent luminance decrease stimuli and then again to a set of three trials with the dark looming stimulus. Responses to each stimulus were videoed and quantified. The inter-trial interval was at least 3 minutes.

All behavioral experiments were conducted at a room temperature of 24-25°C.

Data analysis:

Statistical tests on the hiding responses were performed using SPSS (IBM Statistics, Armonk, NY). When possible, we used ANOVA (one-way and repeated measures) to analyze the data. When unequal variance between groups was found we used Welch's Robust ANOVA.

RESULTS

Development of the LGMD neurons

To study the morphology of the LGMD1 and LGMD2 neurons in all the post-embryonic stages of locust development we used a new neuronal reconstruction approach. We used Neurolucida software for making three-dimensional reconstructions but instead of using it to reconstruct a cell from a single stained preparation, we used it on generally stained (Bodian) sections. Even though this reduced silver method is unspecific, some parameters, such as the copper concentration, can be increased to stain only a small fraction of the neuronal processes

including usually wide-field tangential neurons (Sztarker et al., 2005). Under these conditions, the main and most characteristic branches of the LGMD1 (belonging to subfield A, see below) and of the LGMD2 in the lobula were easily identified and distinguished from each other by their location in these preparations (Fig 1A). We began the reconstructions from a 12 μm section in which some of these distinctive branches appeared, drew the branches contained in all the possible focus planes through that section, using a motorized z drive to record the depth of each feature in the section, and then reconstructed the rest of the neuron following the continuation of each branch, in consecutive sections. When moving from one section to the next external cues (e.g. the border of the neuropils, tracheae, etc) were used to reorient the drawing. If there was no obvious continuity between branches, we still drew the candidate branches and decided afterwards if they belonged to the dendritic tree. In those cases, we either erased the branch afterwards or used the editing tools from Neurolucida to join branches if it was decided that they belonged to the same neuron. This decision was based solely on the proximity of the beginning of the unattached branch to the rest of the dendritic tree (in x, y and z axes) as viewed in the 3D rotating model produced by the software. Supplemental movie 1 shows an example from an ongoing reconstruction of a 4th instar LGMD2 in which the three unattached green branches are evidently part of the same dendritic tree while the red ones belong to a different neuron.

Due to the physical separation between the two neurons, in most cases both LGMD1 and LGMD2 could be reconstructed at the same time. With this painstaking procedure even small branches (about 0.5 μm) could be assigned to a neuronal tree (Fig 1B). Because there are already numerous accounts showing the morphology of the LGMD neurons in adults (e.g.: O'Shea and Williams, 1974; Rind, 1987; Simmons and Rind, 1997; Rind and Simmons, 1998; Peron et al., 2007) and because as the animal grows neuronal reconstruction becomes more demanding and difficult with this method, we didn't include adults in our study. Nevertheless,

our 5th instar reconstruction of the two LGMDs have very similar morphological features when compared with the same neurons intracellularly stained in an adult (Fig. 2A,B). Several reconstructions were made for each neuron type and instar. In the present analysis we considered only the ones where the complete fan-shaped tree was reconstructed (one to three in each category). The reconstruction method was reliable since comparing the morphology of two different reconstructions in different locusts of the same developmental stage rendered similar results (Fig 2C,D). Examples of 3D reconstructions of LGMD neurons seen in different views in a 2nd, 4th and 5th instar are shown in figure 1C-F (Supplemental material movie 2-3). In the adult locust, intracellular staining had previously shown that the arborization of the LGMD1 in the lobula consists of three different dendritic subfields, the most prominent is the fan-shaped subfield A which receives excitatory inputs from medulla columnar elements, the other two, sub-fields B and C, receive inhibitory inputs (O'Shea and Williams, 1974). The LGMD2 possesses one fan-shaped dendritic field in the lobula, located almost parallel but closer to the surface of the brain, and surrounding the subfield A of the LGMD1 (Rind, 1987; Simmons and Rind, 1997; Rind and Leitinger, 2000). In most cases we were able to reconstruct the subfield B of the LGMD1 (Fig 1D-E). The subfield C was more elusive (but see Fig 1E) probably because of the orientation of the thin and long process that connects it to the principal tree that make it difficult to follow through the sections. For the subsequent quantitative analysis we took into account only the subfield A of the LGMD1. An example of a complete reconstruction of the fan-shaped tree of an LGMD1 and an LGMD2 is displayed in figure 3 for each developmental stage. The overall shape (outline) of the LGMD1 and the LGMD2 is similar in all instars. In the two neurons, the fan-shaped arborization is made up of several principal branches of wide diameter arising most of them directly from the principal neurite (they are numbered in the 5th instar examples in Fig 3). Each principal branch gives rise to extensive arborization that occupies a substantial portion

of the dendritic field. The number of principal branches in an LGMD was established on examining the 3D model of the neuron. The number obtained was comparable in all juveniles (Fig 4A) and similar to the number described in adults (7-10 dendrites; O'Shea and Williams, 1974; Simmons and Rind, 1997). Nonetheless, trees do become bigger and more complex as locusts grow. We used the NeuroExplorer tools to quantify this. We chose parameters that have been used before to quantify dendritic changes through development (Lieberstat and Duch, 2002). We used a "color by branch" analysis in which each segment is colored according to its centripetal order in the tree (each order uses a unique color, see Fig 4B; a segment is defined as a portion of a branched structure that is delimited by nodes or terminations with no intermediate nodes). We analyzed the length and order of each segment in a tree. Larval development was accompanied by an increase in the total length of segments in the fan-shaped arborization (Fig 4C). As can be seen in figures 4D-E, this increase was mainly due to an increase in the number of segments since the mean segment length did not change dramatically with instar stage. To compare the overall shape of the trees we made a branch order analysis showing the distribution of segments in the different branch orders (Fig 4F). In both LGMD neurons, 1st to 4th instars showed a similar distribution of branches, changing mainly in the number of branches in each order. On the other hand, 5th instars showed a wider distribution and a shift toward branches with higher orders. This same tendency is shown in figure 4G displaying the maximum order reached by the branches of the LGMD1 and the LGMD2 in each developmental stage.

Data derived from the segment analysis of the LGMDs, obtained using NeuroExplorer, were also used to assess the developmental differences in the structure of the neurons in a systematic way using Canonical Discriminant Analysis (CDA). CDA is used to find the maximal co-linearity between a set of variables that predicts group membership (Rencher, 1992; Wright et al., 2005). CDA revealed that the ratio of the structural features of the

LGMD1 and 2 neurons could be used to identify which instar the neuron belonged to (Table 1). For each neuron, we obtained three discriminant functions (Table 1, second row), each a combined linear weighting of the anatomical measurements. Taken together, these functions separated LGMD1 and 2 neurons of each of the 4 instars into statistically significant categories that corresponded to the instars (see below). For the LGMD1, the first discriminant function of the CDA explained 92.7% of the variance in the data (Table 1, left columns). The biggest differences for the LGMD1 neuron occurred at the transition from 4th to 5th instar. The 5th instar LGMD1 neuron was the most distinct and was easily separated from all the other instars in the CDA by information about the negative relationship between the Y and Z coordinates of their branch points and by the sign of discriminant function 1 (Table 1, CDA: $\chi_{18}^2=4037$, $p<0.001$). The second discriminant function separated the 1st instar from the 2nd and 4th instars using neuronal branch order as the most important morphological trait for this separation (Table 1, CDA: $\chi_{10}^2=466$, $p<0.001$). The 2nd and 4th instar LGMD1 neurons could be distinguished simply by the correlation between the Y and Z coordinates; in the 2nd instar, there was a greater difference in Y and Z than in the 4th instar (Table 1, CDA: $\chi_4^2=175$, $p<0.001$).

The LGMD2 neurons (Table 1, right columns) also grew more distinct as the locusts aged. The 5th instar LGMD2 neuron was separated from all the other instars in the CDA by the first discriminant function with its strong relationship between the X and Y coordinates of each branch point, and with branch order (Table 1, CDA: $\chi_{18}^2=2534$, $p<0.001$). The second discriminant function separated the 4th instar from the 1st and 2nd instars using a combination of morphological features, with only the Z angle not being important (Table 1, CDA: $\chi_{10}^2=1332$, $p<0.001$). The 1st and 2nd instar LGMD2 neurons were distinguished mainly by the higher branch order in the 2nd instar neurons and the XY angle (Table 1, CDA: $\chi_4^2=374$, $p<0.001$).

Of the two neurons, the LGMD1 showed the greatest change in discriminant function 1 between early and late instars, which suggests that, the LGMD1 was initially more rudimentary than the LGMD2 and less like its final form.

While making the dissections needed for this study we noted that the brains of young locusts are bigger in relation to their body size than those of adults and young animals also have less fat, connective tissue and muscle surrounding the brain in the head capsule. The same principle is observed if considering the size of the LGMDs. We compared the dorso-ventral extent of the fan-shaped dendritic tree relative to the length of each juvenile locust (Fig 4H). If we compare a 5th and a 1st instar, the size of the animal increases about 4.2 times while the width of the LGMDs only increases about 2.8 times (LGMD1: 2.61; LGMD2: 2.98). As we draw the borders of the lobula in each of the sections that makes up a reconstruction, we can confirm that the surface covered by the neurons with respect to the neuropil is similar in all instars.

Hiding behavior

We recorded the responses of 10 animals from each larval stage to an approaching black ball (Fig 5; see details in Materials and methods). During each trial, locusts were filmed from above. The type of behavior triggered by the looming stimulus was sorted into three categories: hiding, startle and no response. A hiding response involved a re-positioning behavior in which locusts sitting on a rod turned to hide from an approaching stimulus by moving behind the object. A complete hiding response involved a turn through 90 degrees, to a position in which the rod ended up between the locust and the ball. A startle response consisted of a short, slightly lateral, sudden movement. Some juveniles quickly moved back to the starting position when displaying this response, so the final rotation angle was usually very small or even zero.

In a previous report, 5th instar locusts were found to perform different intensities of hiding responses depending on the angle with which the stimulus was presented and on parameters such as velocity, contrast and room temperature (Hassenstein and Hustert, 1999). Here, we compared locusts' ability to display escape responses throughout larval development and, therefore, we used only one stimulus (with a fixed velocity, size, color, and direction) to present to all animals. We observed hiding responses in all instars, including 1st instars, less than 1 day old (Fig 6A). However, some of the younger instars (1st to 3rd instars) sometimes showed a startle response instead. This is likely to be a behavioral choice rather than a developmental constraint since animals displaying startle responses could hide in other trials. Startle responses were not seen in older animals (Fig 6B). Since the hiding response is performed by all nymphal stages, the following question arises: are the characteristics of such a response similar in all stages of development? By exploring different parameters of the hiding response we discovered changes in latency and rotation velocity but not angle of movement (Fig 6C-E). The latency of response changed significantly with developmental stage (Welch Robust Anova: $F(4; 21.58)=2.658$; $p=0.035$) becoming shorter as locusts grow and reaching a minimum value for 4th instars. This difference meant that the youngest instars responded to the approaching object later than the older instars, when it subtended a larger angle on the retina but always before the end of the approach (see Fig 6C). The angle rotated during the hiding response showed a tendency to increase with age, but instar was not a statistically significant factor (Fig. 6D; One way Anova: $F(4; 42)=2.423$; $p=0.063$). On the other hand, instar had a significant effect on the initial angular velocity of the responses, with the younger instars slower than 4th instars and adults (Fig 6E; Welch Robust Anova: $F(4; 20.396)=3.792$; $p=0.019$).

The movement of the real ball is subject to acceleration due to gravity and generates non visual cues (e.g. mechanical stimulation produced by the movement of the air displaced by

the ball). These effects are especially strong in the final part of the approach when younger instars respond. To verify that young instars were using vision to detect the approaching stimulus and to gain more information about hiding responses through development, we used computer-generated looming stimuli. We compared the responses triggered by dark and light looming disks of the same contrast against background in 1st, 3rd and 5th instars. Computer-generated stimuli were less effective than the real ball in evoking hiding in the locusts and we had to use a slower approach to evoke conspicuous responses (see methods). Even with this stimulus, a proportion of animals responded to the looming stimulus with a startle response, or did not respond at all (Fig 7A). This was the case even for 5th instars that had previously responded by hiding when stimulated with the real ball.

Despite this, the experiment clearly shows that a different behavioral output is obtained when using dark or light stimuli. This difference was consistent across instars since a similar pattern of distribution of responses was observed in all instars (Fig 7A). In particular, we found that locusts performed a hiding response more frequently when stimulated with a dark object than with a light one (Supplemental material movie 4-5). In contrast, most of the trials using the light stimulus elicited no response (Supplemental material movie 6-7). On the other hand, the contrast sign of the stimulus did not seem to systematically affect the probability of displaying a startle response (Fig 7A).

Hiding responses were evoked exclusively by the looming stimulus, in which edges moved, since no hiding responses were recorded when an equivalent luminance change stimulus, with no edge movement, was used (Fig 7B). To test this lack of response was not simply due to habituation we recorded the responses of nine 3rd instar locusts to stimulation with a dark looming stimulus (loom 1), then an equivalent luminance decrease stimulus, and then another dark loom (loom 2). We divided the responses into three categories: hiding, startle and no response (Fig 7B). The dark looming stimulus (loom 1) triggered mainly hiding but also

startle responses (first panel Fig 7B), giving a median overall turning angle of 20 degrees. In contrast, no hiding and only one startle response was recorded to the luminance change presented alone (second panel Fig 7B), giving a median turning angle of 0 degrees. Loom 2 triggered mainly hiding responses (third panel Fig 7B), with a median overall turning angle of 10 degrees. The angular extent of the hiding responses to looms 1 and 2 were not statistically different (Mann-Whitney Rank Sum Test on non-normal data, $p=0.727$) but they were both significantly different from the response to luminance decrease alone (Mann-Whitney Rank Sum Test on non-normal data, $p<0.001$), indicating that the lack of response in the luminance decrease trials was not due to fatigue or habituation but due to the stimulus itself.

Further evidence of the different releasing strength of the dark and light stimuli, is that in general, in trials where hiding occurred, the response was stronger (larger angle of response) when the dark object was used (Fig 7C).

We found a consistent difference in the latencies of the hiding responses triggered by the dark and the light stimuli. Such difference was not present when comparing startle responses. In fact, in all instars tested, the latency of the hiding response was significantly shorter when the dark stimulus was used (Repeated measure ANOVA, Instar factor: $F(2;9)=3.563$, $p=0.072$; Stimulus factor: $F(1;9)=24.07$, $p=0.001$; interaction $F(2;9)=3.058$; $p=0.097$; Fig 7D). Taking all instars together the response latencies were 5.27 ± 0.08 sec (corresponding to a subtended angle of 24°) for the dark stimulus and 5.69 ± 0.05 for the light stimulus (corresponding to a subtended angle of 38°).

DISCUSSION

Holometabolous insects, such as flies and moths, change radically both in shape and in behavior during development. The crawling larva molts into a pupa which over the space of a few days gives rise to a fast flying adult. This implies a complete remodeling of the CNS, involving drastic changes in the intrinsic properties, dendritic architecture and synaptic interactions of neurons (reviewed in Consoulas et al., 2000; Liebersat and Duch, 2002, Meseke et al., 2009). In contrast, the development of hemimetabolous insects, such as the locust, is gradual; first instars' morphology and physiology are very similar to the adults' with only a few distinctions (i.e they can't fly like the adults). The question is then, are the brains of 1st instars also small, miniature but functional versions of the adults'? Or do new networks controlling adult behaviors only develop, when the behaviour is actually displayed? Evidence of the former comes from the work of Dagan and Volman (1982) in which they show that first instar cockroaches, with only 2 wind-receptive filiform hairs in each cercus (compared with 200 in the adult) are still able to perform accurate escape responses comparable to those of adults. In locusts too, Bucher and Pflüger (2000) showed that the wind-sensitive interneuron A4I1 has the same general morphological features in all developmental stages and that directional sensitivity is already present in 1st instars. In the locust visual system, the DCMD looks very similar in embryos and adults, undergoing only allometric enlargement (Bentley and Toroian-Raymond, 1981) and is functional in the first instar (Simmons et al., 2013).

There are also no hard and fast rules about the development of adult specific behaviour and neuronal circuits. Fully functional neural circuits involved in adult-specific motor patterns have been documented in newly hatched locust nymphs. For example, flight motor activity could be evoked in 1st instar locusts' motor nerves using direct application of octopamine to the thoracic ganglia although the animals are not capable of responding to a wind stimulus with a flight motor output (Stevenson and Kutsch, 1986). Another example in locusts are

three auditory neurons (G, B and C) that show the same general morphology, dynamic properties and synaptology in early instars and adults despite the fact that 1st instars are probably deaf to airborne sound (Boyan, 1983). In contrast, circuits involved in song production and flight in crickets develop gradually during successive molts but are not functional at hatching (Bentley and Hoy, 1970). To summarize, some 1st instars are born with the nervous system ready to perform particular adaptive behaviors accurately while circuits involved in the adult behavioral repertoire develop progressively, and might be subject to silencing by descending inhibition during nymphal stages (Anton et al, 2002; Bentley and Hoy, 1970). Therefore, the maturity of the underlying neural circuitry and the ability to display a behavior are not necessarily correlated. Taking that into account, we studied the ontogeny of escape behaviors triggered by approaching stimuli and of key neurons that could be involved in such responses in *Locusta migratoria*.

Ontogeny of the LGMDs

The capacity to detect looming objects and to act rapidly and appropriately is crucial for the survival of many animals. Most studies have focused on the structure and function of adult neurons even though collision-avoidance behaviors have been described from the start of an animal's life (e.g. human babies, Bower et al., 1971) and are probably innate or require minimal learning (Fotowat and Gabbiani, 2011). Here, we have described the LGMD1 and the LGMD2 neurons in each larval stage of the locust (even in 1st instar locusts that are about 6 mm long). We combined a classical histological method with a powerful system for three-dimensional neuronal reconstruction so the resulting reconstructions had a quality and detail that allowed an analysis of the changes in the neuronal structure during development. The dramatic shape and morphological constancy of the LGMD neurons (Rind and Simmons, 1998; Rind and Leitinger, 2000) made this approach feasible. The basic structure of the

LGMDs seems to be already laid down at hatching since, for example, the number of principal branches was constant throughout development for both neurons and the outline of the neurons was similar in all the instars. We found an allometric enlargement of both dendritic trees during larval development. As was mentioned before, a similar pattern has been described for the DCMD neuron that is postsynaptic to the LGMD1 (Bentley and Toroian-Raymond, 1981), and for other identified neurons (Blagburn and Beadle, 1982; Shankland and Goodman, 1982; Boyan, 1983). However, it is interesting that for the LGMDs, the increase in total length observed in the dendritic structures during development was mainly due to an increase in the number of segments and not to an increase in the mean segment length (Fig 4C-E). We couldn't find studies in hemimetabolous insects with detailed enough information about individual neurons to compare these data with. In a holometabolous insect, the moth *Manduca sexta*, motoneuron 5, which in the larva innervates a crawling muscle and in the adult a wing, has two different modes of dendritic growth during its pupal to adult development. The first is a rapid, growth-cone-dependent phase in which new branches are added at order 1 to 40. In this period, the changes in total dendritic length are correlated with strong changes in the number of segments but with minor changes in the average dendritic segment length. The second phase is growth-cone-independent and involves a slower branching, limited to high-order dendrites and to the perimeter of the dendritic field (Lieberstat and Duch, 2002). Even though the development of hemimetabolous insects, such as the locust, is gradual (in contrast to holometabolous post-embryonic development with its extensive dendritic regression and then re-growth) two similar phases were evident in the development of the LGMD1 and 2. Like phase 1 in moths, there was a gradual growth in the LGMDs dendrites during the first 4 larval stages and all instars had branch orders with similar frequency distributions and only minor changes in the highest branch order. In 5th instars there was a large increase in the highest branch order and a shift in

the frequency distribution of branch order towards higher orders as found in phase 2 in the moth (Fig 4F-G). A large change in dendritic development between 4th and 5th instars was also confirmed using a Canonical Discriminant Analysis.

Interestingly though, in locusts the growth of the dendritic trees does not decelerate between 4th and 5th instars, showing a rather constant (or even exponential) increase during all developmental stages that would fit a phase 1 type of growth. We think that this phenomenon is related to the progressive addition of new ommatidia that occurs during post-embryonic development and it would involve the concomitant addition of new segments in which the new connections would be established. New ommatidia are added at the anterior edge of the eye and become functional after each moult (Anderson, 1978). In *Locusta migratoria*, the number of ommatidia increases from 2200 in 1st instars to about 8100 in adults (Simmons et al., 2013). Consistent with that, the structural parameter that best separated the morphology of the LGMD1 and LGMD2 in the different instars in the CDA was the Y coordinate of each branch point in the main dendritic tree (first discriminant function, table 1). This coordinate, is most closely correlated to the position of the tip of the branches of the fan-shaped arborizations as they develop and has been shown to receive information from the anterior part of the retina in the LGMD1 (Peron et al., 2009), precisely the region where new ommatidia are added in each moult.

In line with the results discussed in this section, we have recently shown that the DCMD neuron from a 1st instar is already capable of distinguishing approaching from receding objects, and of discriminating between different approach speeds (Simmons et al., 2013). However, the responses are refined considerably throughout successive instars: the DCMD becomes more selective in its response and the vigor of its responses to looming objects increases, both of which might also be partly explained by the increase in the size and number of ommatidia in the eye.

Brain, behavior and development

To examine the ontogeny of escape behaviors evoked by approaching stimuli we analyzed the responses triggered by real and computer-generated looming. All locusts responded readily to an approaching dark ball, including those only a few hours post-hatching. Differences in the probability of displaying startle or hiding responses were evident between younger animals and adults, with more hiding responses shown by adult locusts (Fig 6).

Certainly, the real ball also stimulates other sensory modalities apart from vision (e.g. mechanoreception). These non-visual cues become stronger as the ball approaches the locust coinciding with the time younger instars start responding. Therefore, we verified that indeed young instars were employing vision to detect the approaching stimulus by using computer-generated stimuli. We tried different velocities and sizes to find the more effective stimulus for triggering hiding behavior. No consistent responses were evoked when using a virtual stimulus with a similar $1/|v|$ to the real ball. The more effective stimulus turned out to be the slowest one we tried with an $1/|v|=300$ msec, but even with this stimulus we found a greater proportion of trials with no response using the virtual images. Interestingly, and against our predictions, we obtained a similar probability of performing hiding and startle responses in 1st and in 5th instars. Even though many attributes of the stimuli (velocity, sensory modalities targeted, etc) are different between real and computer-generated looming, it is notable that the relative efficacy of the stimuli for triggering hiding responses changes between instars: for 5th instars the dark projected stimulus was less salient than the real black ball, since they displayed startle or no responses in 40% of the trials while for younger instars, the computer-generated stimulus was more effective than the real ball (1st instars increased their probability of hiding from 44 to 57% and 3rd instars increased from 81 to 90%). These differences could be explained by the increase in optical resolution that occurs during development (Simmons

et al., 2013). The poorer visual acuity might also explain the delayed hiding responses observed in younger instars (Fig 6C).

Both hiding and startle responses were performed by all nymphal stages although with probabilities that depended both on the locusts' age and the stimulus used. The particular escape strategy selected was not set by developmental constraints, as animals displaying startle responses could hide in other trials. We did, however, find ontogenic changes in the hiding response: its latency got shorter, and its velocity increased, with locust age. Nonetheless, these kinds of changes do not require the presence of new mature elements in the circuitry controlling the response and are consistent with a refinement of neurons and connections already present at hatching.

Are the LGMDs involved in hiding behavior?

Hiding is a robust evasive response displayed by locusts and other grasshoppers to looming stimuli (Hassenstein and Hustert, 1999). The neural pathway generating this response is unknown but since it is triggered by looming stimuli, the LGMDs are good candidates. The neural reconstructions of the LGMDs show that their structure is identifiable in 1st instars though the LGMD2 seems more mature than the LGMD1 since it is the bigger in size (segment number, length, LGMD extent/locust length ratio) and complexity (branching pattern, highest branch order). CDA analysis also supports this maturity of the LGMD2 at hatching when, of the two LGMDs it is closest to the 5th instar neuron in structure. However, we know from physiological experiments that the LGMD1 (read through the DCMD output) is capable of detecting approaching stimuli in newly hatched instars (Simmons et al., 2013). Therefore both LGMD neurons are potential candidates for being involved in the hiding response circuit. The experiments performed with virtual stimuli revealed very few hiding responses to the light stimulus but strong and consistent hiding responses in all instars to the

dark stimulus (Fig 7). The number of trials without a response was very high when using the light stimulus but low when using the dark one. In the adult, a difference in the stimulus preference of the LGMDs has been reported: a light looming stimulus does not excite the LGMD2 but does excite the LGMD1 (Simmons and Rind, 1997). If such response preferences were similar in younger instars, then the clear difference in the effectiveness of the two stimuli would suggest a role for the LGMD2 neuron in triggering the hiding response. Further experiments both in juveniles and adult locusts will be necessary to prove such a hypothesis.

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FIGURE LEGENDS

Figure 1: Three-dimensional neuronal reconstructions of the LGMD1 and the LGMD2 from Bodian-stained brains. A) Photo-montage of a lobula (2nd instar) stained with Bodian's method obtained from images of 5 consecutive 12 µm sections that were made transparent, aligned and then flattened in Photoshop. B) Reconstructions derived from the same preparation as A, made using Neurolucida. To aid understanding, points belonging to the LGMD1 (white arrowheads) and to the LGMD2 (black arrowheads) that correspond in A and

B are marked. C) Stereo image of the same reconstructed neurons showed in B but rotated 180°. D) Three different views of the 2nd instar LGMD1 shown in B where a second dendritic field (subfield B, black arrows) in addition to the main one is evident. E) Six different views of a 4th instar LGMD1 showing the smaller dendritic subfields B (black arrows) and C (white arrows) in addition to subfield A. F) Six different views of the LGMD1 and 2 neurons reconstructed in a 5th instar showing the contiguity of the dendritic trees. Anatomical coordinates defined relative to the head: x: dorso-ventral, y: Proximo-distal, z: antero-posterior. Scale bar: 10 µm in A-C; 50 µm in D-E.

Figure 2: Comparison between a confocal reconstruction of the two LGMD neurons stained intracellularly in an adult (A) and a Bodian based Neurolucida reconstruction of the same neurons in a 5th instar (B). White arrows point to the LGMD1 and green arrows to the LGMD2. C, D) Two examples of the LGMD2 neuron reconstructed from two different 1st instar locusts. Scale bar: 50 µm.

Figure 3: Presence and development of LGMD1 and LGMD2 in all instars. An example of a complete reconstruction of each neuron in all nymphal stages is shown. They are all displayed in a frontal view and at the same scale. X, y and z axes are defined relative to the locust's head. In the 5th instar reconstructions, the principal branches contained in the fan-shaped arborization are indicated by successive numbers. Regardless of minor differences caused by the original orientation of the sections from which the neurons were reconstructed, some of these branches are easily identifiable in all the other instars. Scale bar: 50 µm.

Figure 4: Quantification of changes in the morphologies of the LGMD1 and the LGMD2 during larval development. A) The number of principal branches contained in the fan-shaped

arborization was counted for the neuron reconstructions shown in figure 3. B) An example of the colored by branch order analysis of a 4th instar LGMD2. Each branch is colored according to its centripetal order in the tree (a few are numbered as examples). Scale bar: 50 μ m. C) Total length (calculated as the sum of the lengths of all the branches in a given tree) was analyzed for all the neural reconstructions that were complete (1st-5th instar: LGMD1 n=2; 2; 2; 3; 1; LGMD2 n=2; 2; 3; 2;1). D) Number of segments (defined as a portion of a branched structure that is delimited by nodes or terminations and with no intermediate nodes) and E) mean segment length for each reconstruction. F) With the information derived from the branch order analysis (see B) we constructed histograms for the neuron reconstructions in figure 2 showing the number of segments in the different branch orders. Left: branching pattern for LGMD1. Right: branching pattern for the LGMD2. G) The highest branch order also illustrates the complexity of a tree since numbers are successively assigned to branches to describe the hierarchy of the branching scheme. All the neural reconstructions that were complete were used in this analysis. H) The ratio between the dorso-ventral extent of the fan-shaped arborization of the LGMD and the locust length was calculated for the animals from which the reconstructions shown in fig 3 were made. Bars show mean \pm SE.

Figure 5: Setup for behavioral experiments using a real ball. A) We used a 10 cm diameter black Styrofoam ball that rolled along a 1 meter rail (inclination 2.2°). Locusts sat on the top of a wooden rod proportional to their size (black arrowhead). Two metal rings each containing a motion detector monitored the passing of the ball: soon after release and near the end of the trajectory (white arrowheads). B) Locust's point of view. The ball was set free remotely by the smooth movement of a long stick (black arrow).

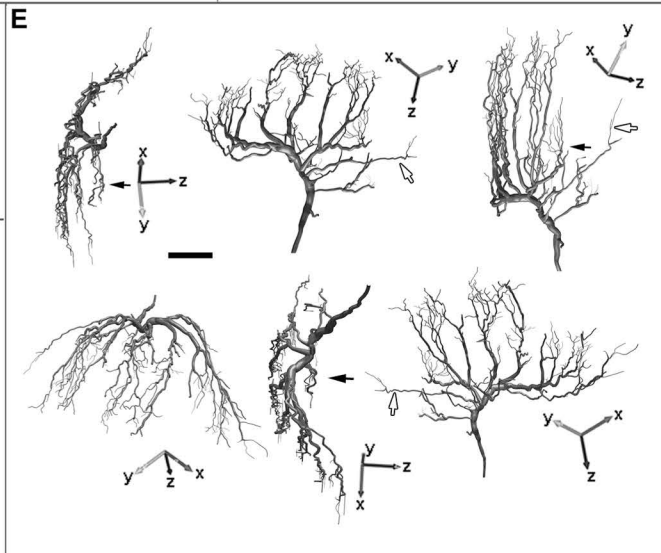
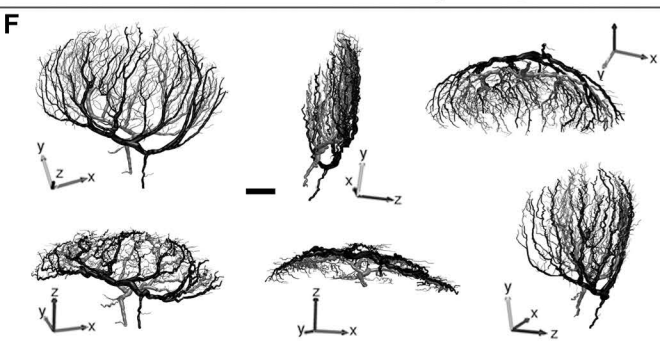
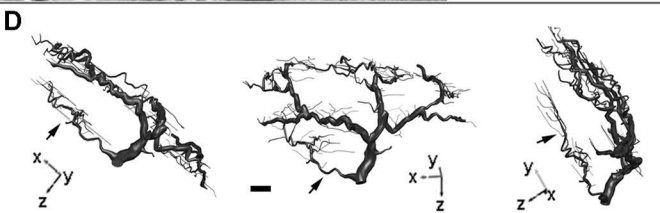
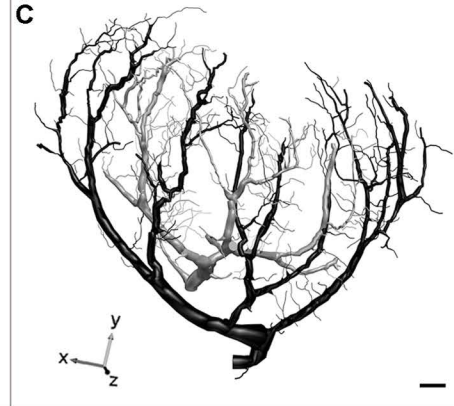
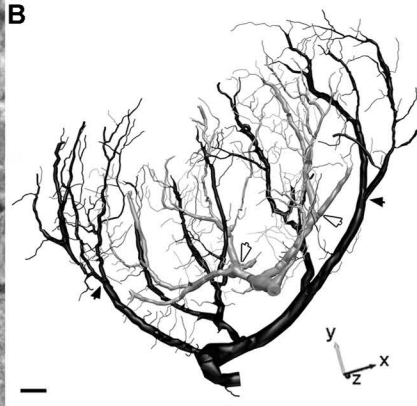
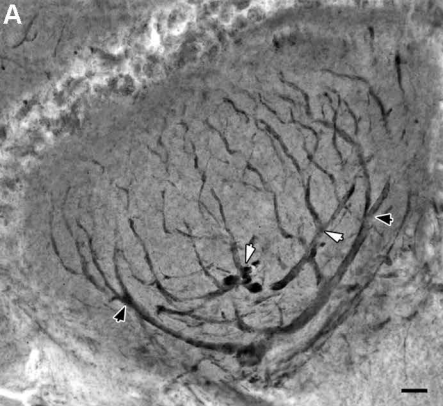
Figure 6: Hiding responses are seen throughout development. A) Examples of hiding behavior performed by all the instars (1st instars to adults). Top view images of the locust's position captured at the start of the trial (left column) and after the end of the hiding response (right column). The ball approached from the top of the image. Scale bar: 1 cm. B) Proportion of responses evoked by the looming stimulus in each stage of development. Black bar: startle responses (St). Gray bar: hiding responses (Hi). Number of trials included 1st-adult: 36, 35, 32, 28, 30, 30. C-E) Parameters of the hiding response. Only locusts that responded with a hiding response were considered. C) The latency of response changed significantly with instar (Welch Robust Anova: $F(4; 21.58)=2.658$; $p=0.035$) decreasing as juveniles grow (pairwise comparisons post-hoc by DMS, $p<0.05$ for 1st vs 4th and 2nd vs 4th). This means that the youngest instars responded to the object at a larger subtended angle (see inset). D) The angle turned during hiding showed a small increase in successive instars but this factor was not statistically significant (One-way Anova: $F(4; 42)=2.423$; $p=0.063$). E) The velocity of response changed significantly with instar stage being the first three developmental stages slower than the older ones (Welch Robust Anova: $F(4; 20.396)=3.792$; $p=0.019$; pairwise comparisons post-hoc by DMS, $p<0.05$ for 1st vs 4th; 1st vs adult; 2nd vs 4th and 3rd vs 4th). Bars show mean \pm SE. Different letters stand for significantly different groups.

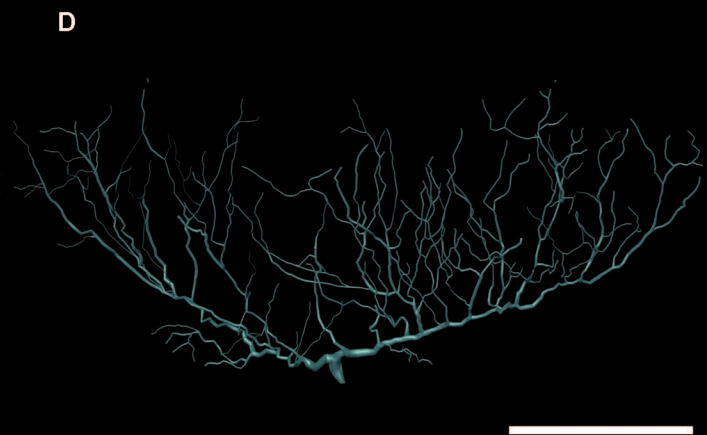
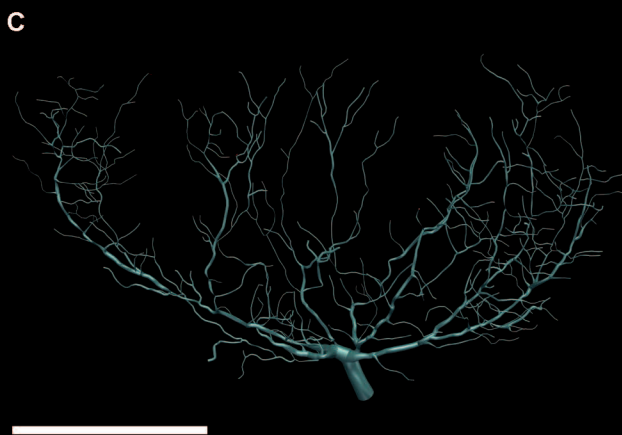
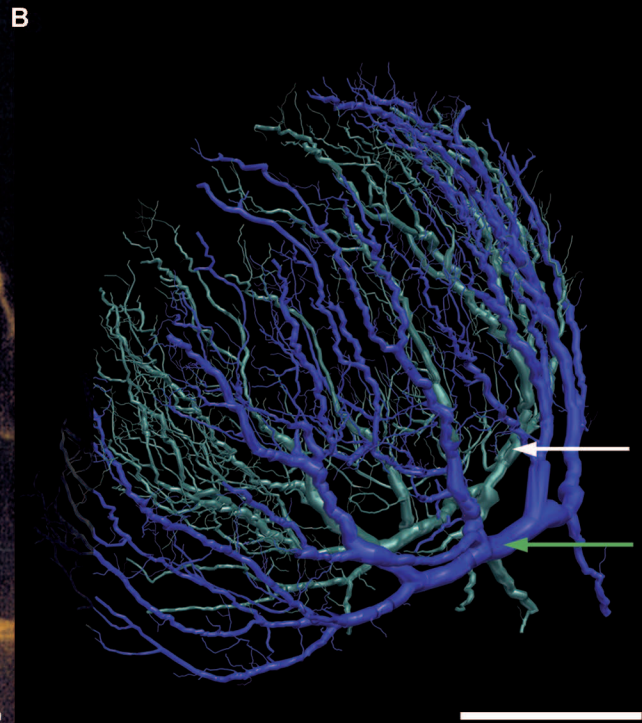
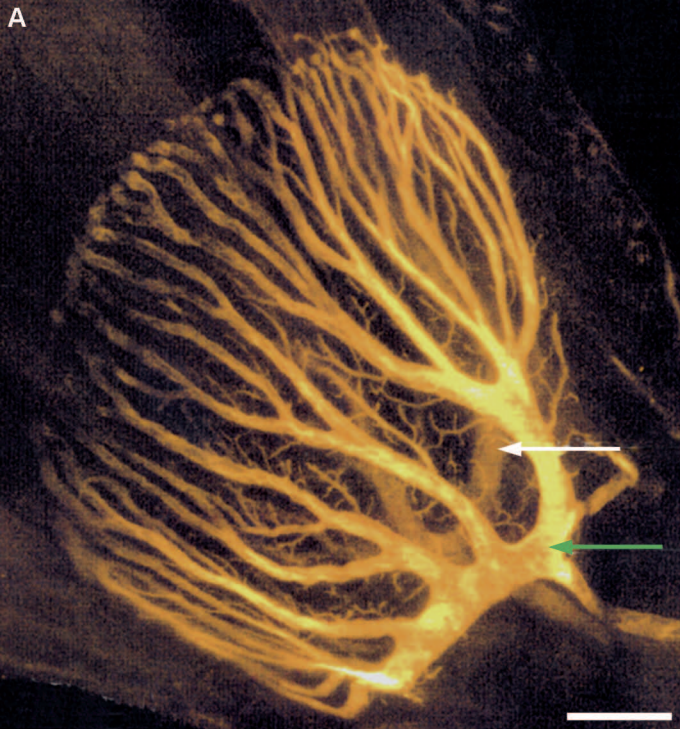
Figure 7: Computer-generated light and dark approaching discs evoke different responses. A) Frequency of performance of different behaviors (no response (NR): black; startle (St): light gray; or hiding (Hi): dark grey) in response to dark and light looms (n=10 locust per instar group, 6 trial repetitions per animal). B) Frequency of performance of different behaviors (no response: black; startle: light gray; or hiding: dark grey) triggered by a dark loom (dark1), by an equivalent luminance decrease stimulus (lum) and by a another dark loom (dark2). C) Intensity of response is represented here by the angle of rotation performed. The number of

trials with responses in each group is depicted in parenthesis. Data for 3rd and 5th instars is shown. D) Latency of response relative to the time of collision. It is measured as the time that the looming ended its expansion minus the frame before a movement of the locust was detected. Each graph displays data from a single developmental stage, the instar group is displayed at the top left corner. Bars show mean \pm SE.

TABLE LEGENDS

Table 1: A Canonical Discriminant Analysis applied to the data for the LGMD1 (left columns), and to the data for the LGMD2 (right columns), allowed us categorize the data as coming from a particular instar. The data consisted of 6 structural measurements from the anatomy of reconstructed LGMD1 and 2 neurons in size matched 1st, 2nd, 4th and 5th instars. The magnitude and the sign of these coefficients indicates the amount of separation between categories and which instar is being separated from the rest by the function.



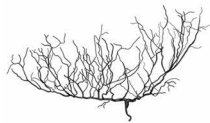


LGMD1



1st instar

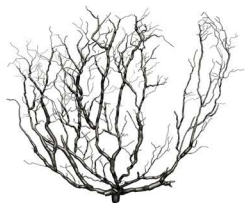
LGMD2



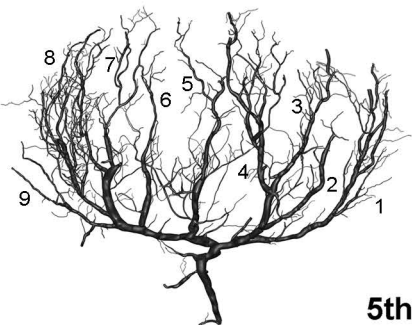
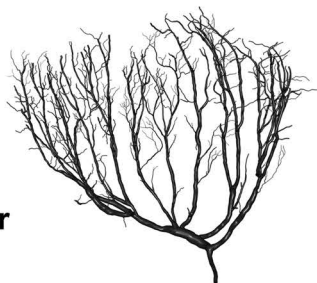
2nd instar



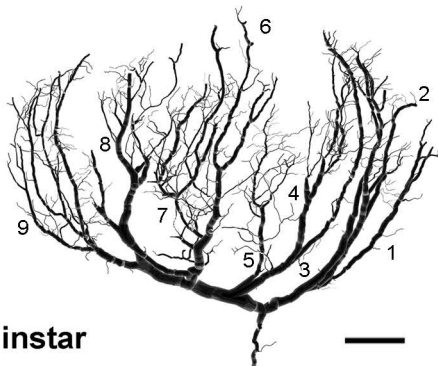
3rd instar

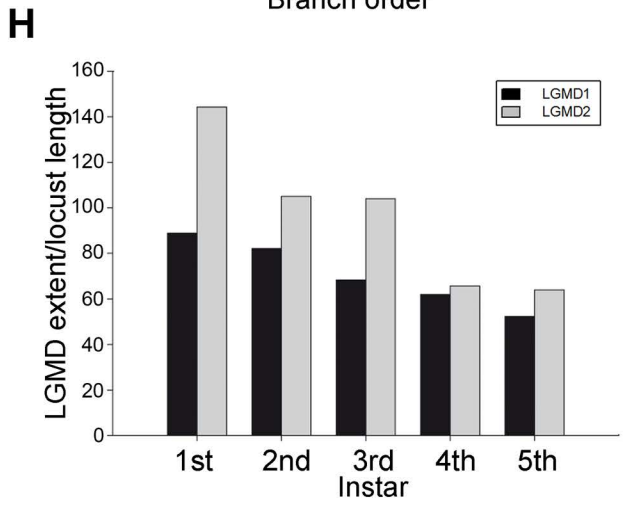
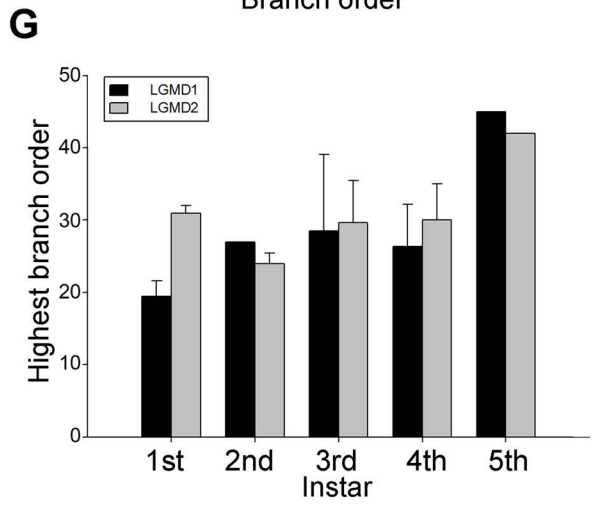
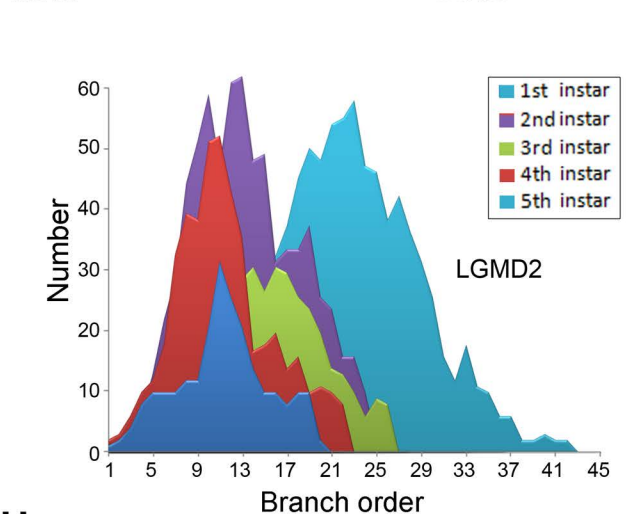
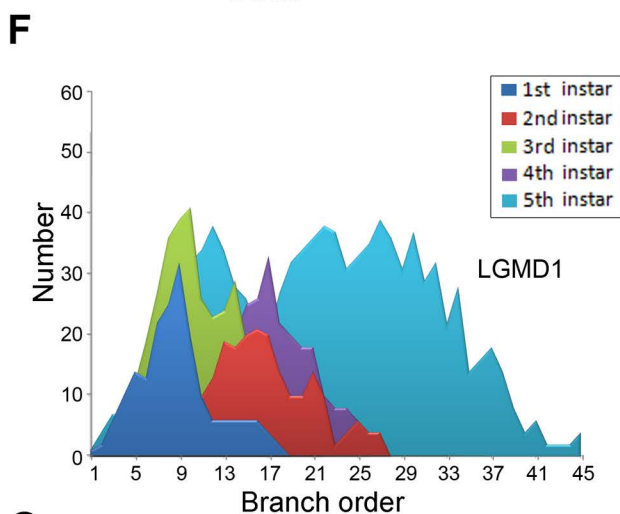
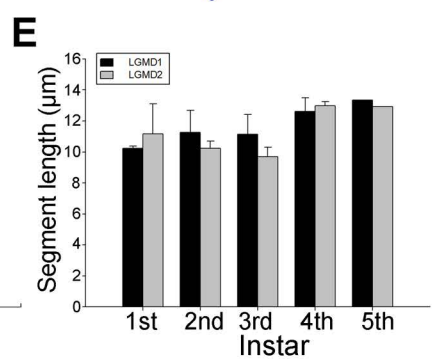
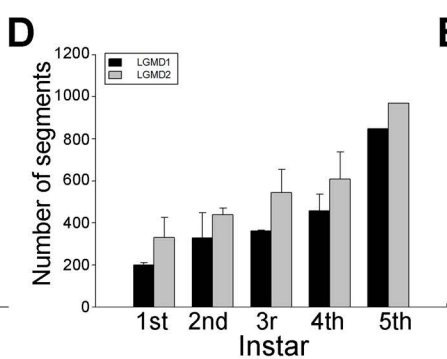
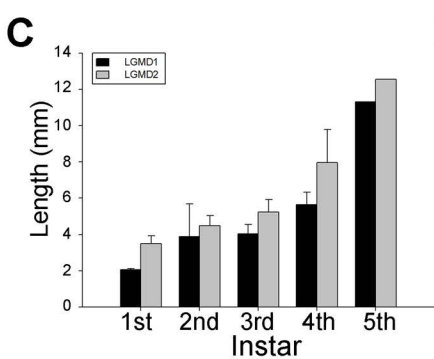
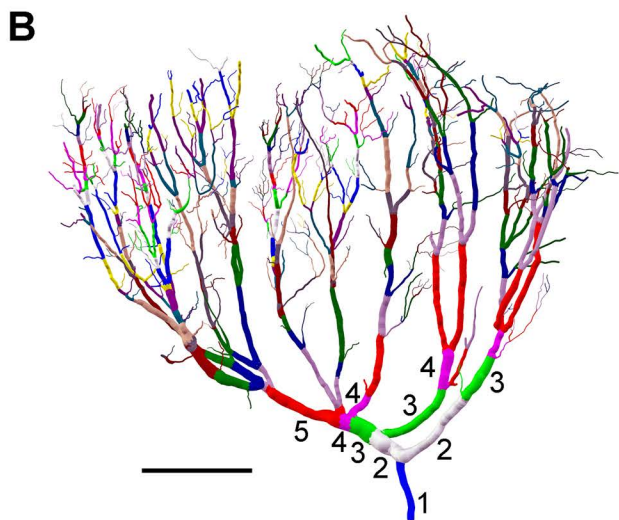
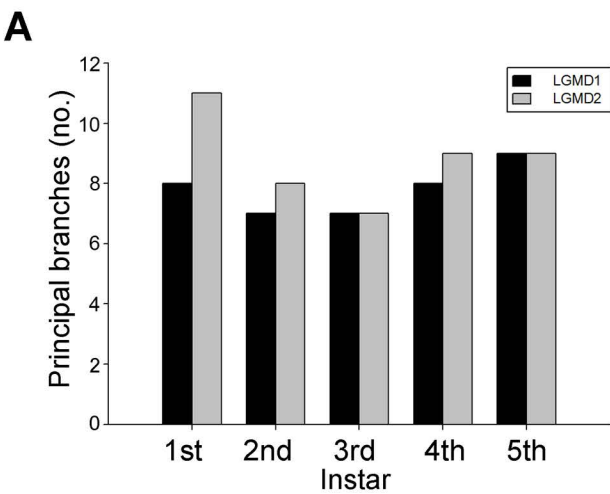


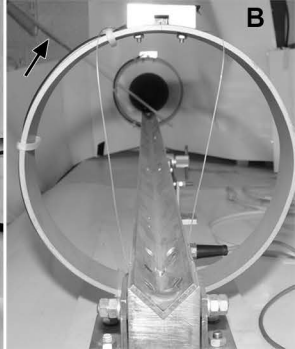
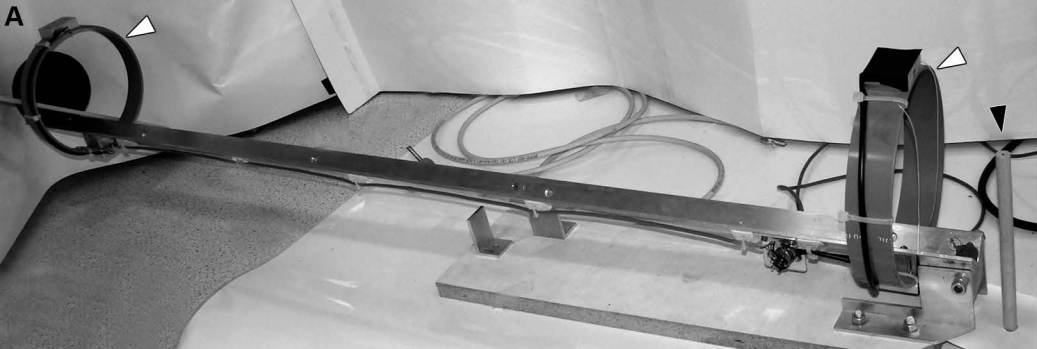
4th instar

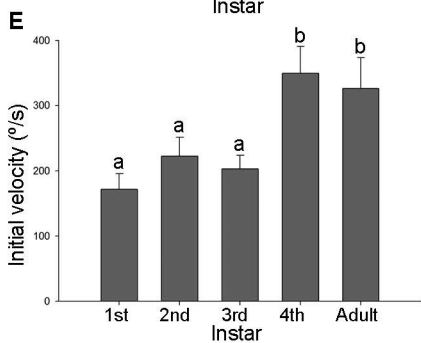
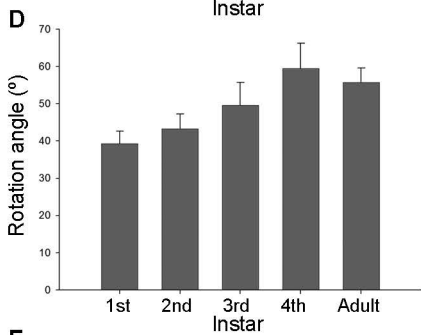
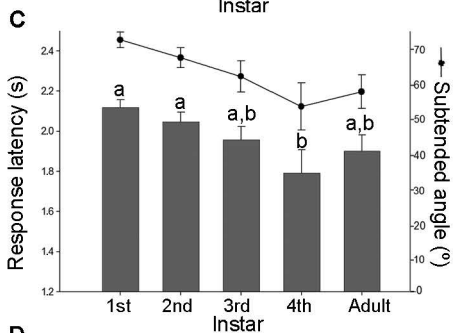
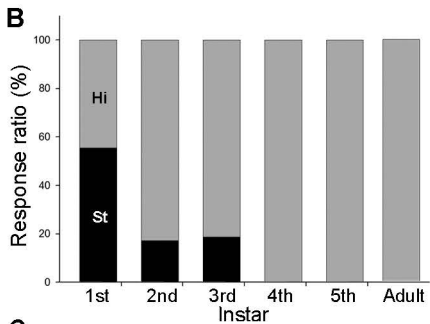
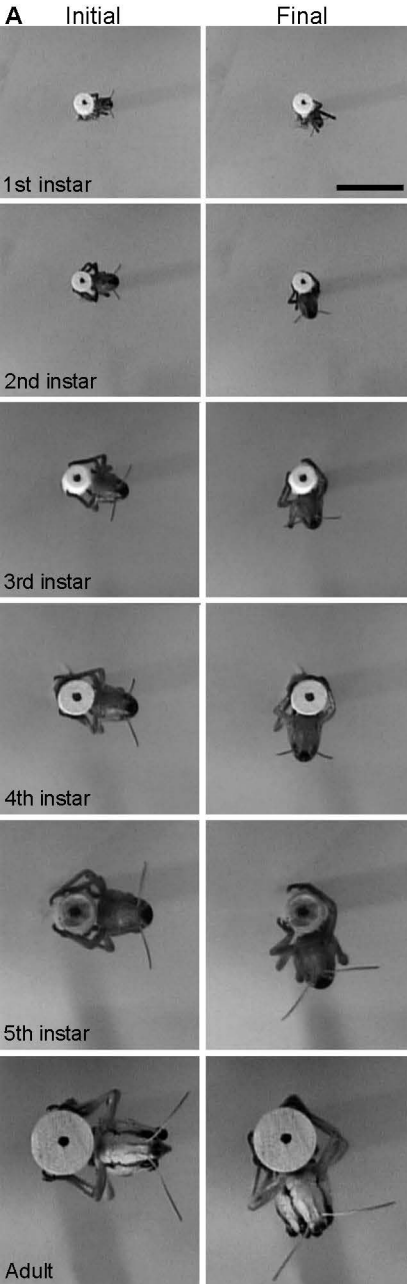


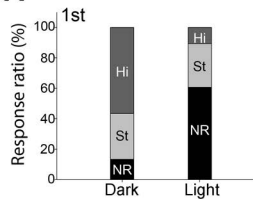
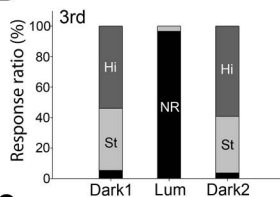
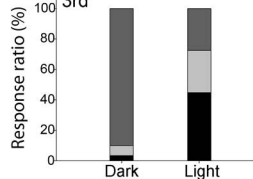
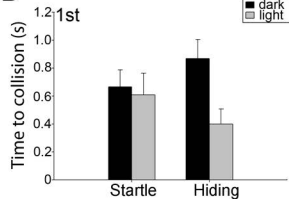
5th instar









A**B****D****C**